Purification, characterization and application for preparation of antioxidant peptides of extracellular protease from *Pseudoalteromonas* sp. H2

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Abstract

The study reported on the isolation of a metalloprotease protease from *Pseudoalteromonas* sp. H2. EH2 maintained more than 80% activities over a wide pH ranges of 5-10 and the stability was also nearly independent of pH. Over 65% of activities were detected in a wide temperature of 20-70℃. The high stability of the protease in the presence of different surfactants and oxidizing agent was also observed. Moreover, we also investigated the antioxidant activities of the hydrolysates generated from porcine and salmon skins collagen by EH2. The results showed that salmon skins collagen hydrolysates demonstrated higher DPPH (42.88%±1.85) and hydroxyl radical (61.83%±3.05) scavenging activity than porcine skins collagen. For oxygen radical absorbance capacity, the hydrolysates from porcine skins collagen had higher efficiency (7.72±0.13 μmol·TE/μmol). Even 1 nM mixed peptides can effectively reduce the levels of intracellular ROS. And the two types of substrates exerted the best antioxidant activity when hydrolyzed for 3 hours. Hydrolysis time and type of substrate exerted important effects on the antioxidant property of hydrolysates. The hydrolyzed peptides from meat collagens by protease have good antioxidant activity may have implications for the potential application of marine protease in biocatalysis industry.

Keywords: *Pseudoalteromonas*; protease; hydrolysis; collagen; antioxidant peptides

1. Introduction

Proteases are enzymes that hydrolyze the peptide bonds of proteins[1]. They are the most important and commonly used in industry, which contribute about 60% of the total market of industrial enzymes[2], such as detergents[2], anti-biofilm agents[3], feather processes, feed processing, wastewater treatment, and food processing[4]. Microorganisms could produce extracellular proteases, high-producing protease strains were easy to culture and genetic manipulation to generate novel enzymes. Therefore, using microorganisms to produce proteases had a lot of advantages, such as a wider range of sources, simple to operate, high performing, cost-effective, and can be industrially produced. Marine is a unique habitat with lower temperature and higher salinity. At present, as an excellent source of enzymes, marine microorganisms have gained considerable attention.

Pseudoalteromonas can synthesize potentially valuable biologically active compounds. They are the predominant protease-producing groups in cultured marine bacterium[5] and show high protease-producing ability in the marine environment[6]. Wu et al. purified an alkaline protease from the extracellular protease of *pseudoalteromonas*, which showed valuable feathers as an additive in laundry detergent and non-toxic anti-biofilm agent[7]. Chen et al. demonstrated that serine proteinase secreted by *pseudoalteromonas* can effectively degrade collagen to prepare antioxidant peptides[8]. However, the application of metalloproteinases secreted by *pseudoalteromonas* is uncommon, especially in the preparation of antioxidant

peptides. Some metallo collagenolytic proteases has alkali-, and salt-resistance. And they also have multiple cleavage sites on soluble collagen. In addition, most of the metalloproteinases derived from *pseudoalteromonas* contain PPC domains. Huang et al. have shown that the PPC domain could play a synergistic role in binding, swelling, and assist with the hydrolysis of collagen[9]. In all, metalloproteinases can efficiently hydrolyze soluble collagen.

Animals' skins are a rich source of collagen with a variety of bioactivities. However, they are often discarded as by-products of the meat processing industries [10] and caused serious waste of protein and environmental problem. Many studies have reported that enzymatic hydrolysis of collagen is an effective way to obtain antioxidant peptides[11, 12]. Most of the proteases used in these researches are commercially available, such as trypsin, pepsin, α-chymotrypsin, papain, protamex and neutral protease. However, there are few reports on non-commercially resistant organic solvent alkaline proteases for antioxidant peptides preparation. Therefore, it is of great significance to search and prepare protease which is stable and active under multiple extreme conditions such as alkaline pH, high salt concentration, wide temperature and organic solvent.

In this study, we purified the metalloproteinase produced by *Pseudoalteromonas* sp. H2 and characterized its enzymatic properties. In addition, the potential application of the protease to hydrolyze low-value protein resources to prepare antioxidant peptides was also explored. This study may have implications for the

potential application of marine protease in biocatalysis industry.

2. Results and discussion

2.1 Screening of strain with high protease-producing ability

Proteolytic activity was evaluated by the abilities of forming clear zones on casein plate. The strain H2 exhibited with high protease-producing ability (Fig1A). Compared with other marine strains, caseinolytic profile of *Pseudoalteromonas* sp. H2 has some advantages in terms of the number and brightness of the strips (Fig1B). Based on the superior matching with *Pseudoalteromonas* sp. CF6-1 (FJ169996.1) by the 16S rDNA sequences (99% identity), this strain was designated as *Pseudoalteromonas* sp. H2. And the 16S rDNA sequences of this strain have been submitted to GenBank (No. KX458245). *Pseudoalteromonas* can synthesize potentially valuable biologically active compounds[3] and show high protease-producing ability in the marine environment[5]. Wu et al. have reported that *Pseudoalteromonas* from seawater exhibited activity and stability when varying the temperature and pH or in presence of surfactants and chelating agent[7]. Therefore, separating and preparing the extracellular protease with multiple tolerances from *Pseudoalteromonas* sp. H2 would be useful for future application.

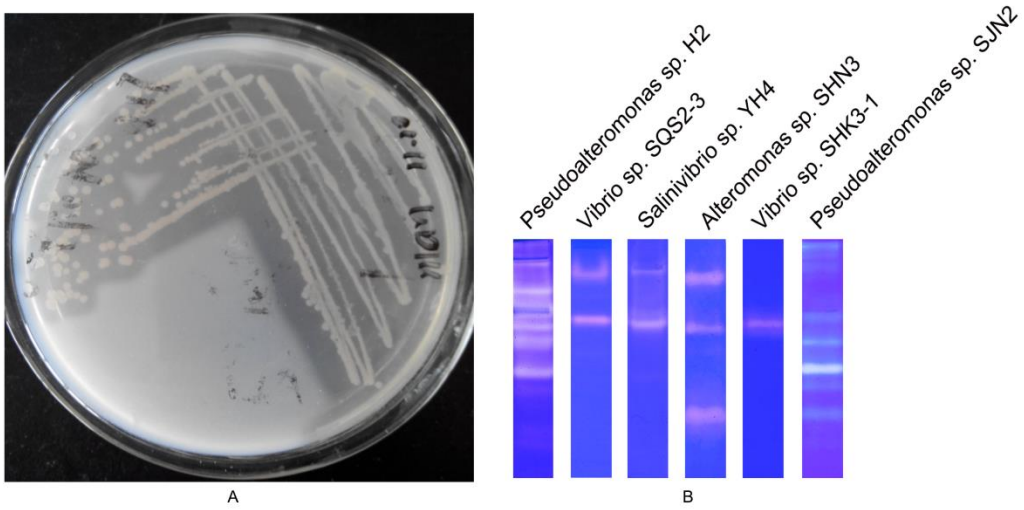

Figure 1. Screening of *Pseudoalteromonas* sp. H2. (A) Forming clear zones on casein plate of strain H2. (B) Comparison of caseinolytic profile from *Ps* sp. H2, *Vibrio* sp. SQS2-3, *Salinivibrio* sp. YH4, *Alteromonas* sp. SHN3， *Vibrio* sp. SHK3-1 and *Ps* sp. SJN2.

2.2 Protease production and purification

Time-related changes of proteases produced by H2 were analyzed by Folin phenol method. In Figure 2A, the strain started to secrete protease in the first 12 hours, and proteases production reached the maximum after cultured for 84 h. After 4 days, the enzyme activity tended to decrease. Therefore, when purifying the protease, the culture supernatant of *Pseudoalteromonas* sp. H2 was collected after 84 h incubation, and purified sequentially with ammonium sulphate precipitation, anion exchange chromatography on HiTrap™ DEAE column, and gel filtration on Superdex 75. The results of purification are shown in Table 1. With yield of 27%, the purified protease has specific caseinolytic activity of 475.2 U/mg. A single band with an approximate molecular mass of 35 kDa was obtained by SDS-PAGE (Figure 2B). Similar molecular masses presented other metalloprotease previously characterized, but the

specific activity of the purified EH2 was higher [13]. Zymography also gave a high degree of consistency with SDS-PAGE analysis. Further confirmed the biological activity and purity of this protease which named enzyme EH2 (Figure 2B).

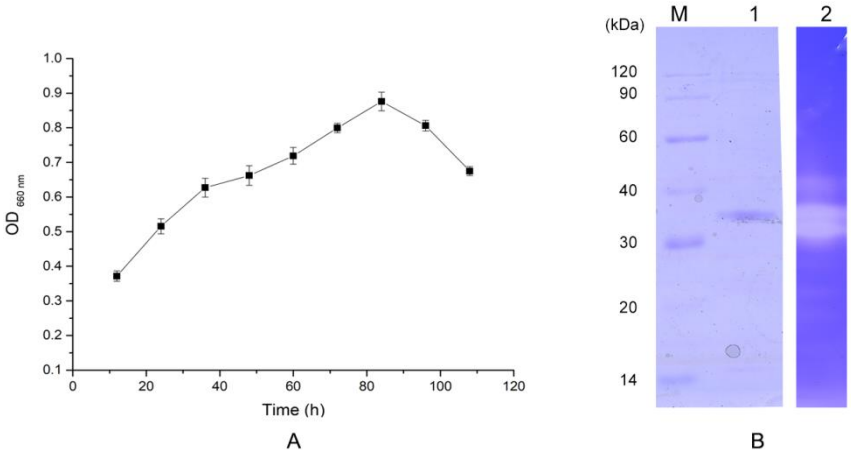

Figure 2. (A) Time-related changes of relative activities of extracellular protease produced by Ps sp. H2. by Folin phenol method. (B) SDS–PAGE and zymography analyses of EH2. Line marked 1: SDS-PAEG; line marked 2: Casein immersing zymography.

Table1. Protease activity in purification process of EH2

Purification stage	Total protein /mg	Total enzyme activity /U	Specific activity (U/mg)
Crude enzyme	350	3678	10.5
Ammonium sulfate precipitation	50.2	2326	46.3
Anion exchange	5.4	1597	295.7
Size exclusion	2.1	998	475.2

2.3 Characterization of EH2

2.3.1 *Effect of temperature and pH on protease activity and stability*

The EH2 was active over a wide pH range. As shown in Figure 3A, more than 80%

activities were detected in a range of pH 5-11, and the maximum activity occurred at pH 7.5-8. This result was comparable to other *Pseudoalteromonas* strains [13, 14]. Furthermore, the stability of the protease was also nearly independent of pH (Figure 3A) and more than 90% of relative activity was exhibited after 24 hours in pH 6-11. The EH2 displays multifaceted potential in different pH and made this enzyme interesting for industrial applications.

Besides that, optimal activity of the protease was observed at 60℃ (Figure 3B). With more than 85% of relative activity at 70℃. Some proteases from *Pseudoalteromonas* also displayed optimum activity at 30℃ [13, 14] or 55℃. This difference was possibly related to the genetic and environmental adaptation of strains. A rise in temperature could enhance the velocity of enzymatic reactions. But the higher the temperature, the more easily the enzyme suffered inactivation. The EH2 displayed a good thermal stability at 50℃ and 60℃ (Figure 3C). The protease maintained more than 65% of its original activity over a wide temperature range of 20-70℃. The EH2 exhibited alkaline-resistance and temperature stability.

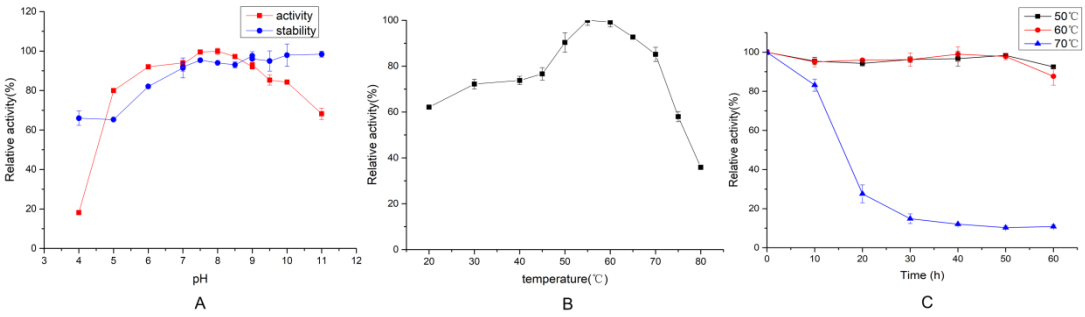

Figure 3. Effects of pH (A), temperature (B) on EH2 activity and thermal stability of EH2 at 50℃, 60℃ and 70℃ (C).

2.3.2 *Effect of inhibitors and metal ions on the protease activity*

The results of inhibitors and metal ions on the EH2 activity were shown in Figure 4. The protease disclosed considerable stability (> 80%) in the presence of Ca^{2+}, Co^{2+}, Mg^{2+}, Ag^{+} and Ba^{2+}. Seawater ions had little effect on protease activity. Mn^{2+} could enhance the activity of EH2 (110-140% relative activity) (Figure 4A). As we all know Mn^{2+} plays a vital role in stabilizing enzyme to prevent thermal degradation [15]. However, the activity of the EH2 was inhibited strongly by low concentration of Cu^{2+}, Fe^{2+}, Zn^{2+} and high concentration of Al^{3+}, Fe^{3+}. The EH2 was sensitive to metalloprotease inhibitor EDTA and OP (Figure 4B). This result indicated that the EH2 was metalloprotease. Similarly, protease activity was slightly inhibited by 10 mM PMSF, showing the involvement of serine residues in the enzyme reaction center.

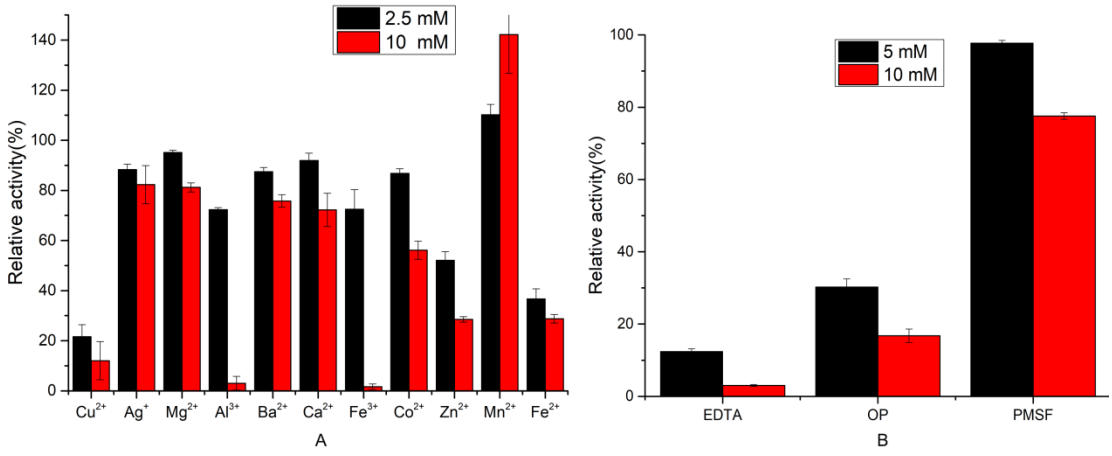

Figure 4. Effects of different metal ions (A) and different inhibitors (B) on protease activity.

2.3.3 *Effect of surfactants, oxidizing agents, and organic solvents on protease activity*

As the media for enzyme reaction, organic solvents exerted important advantages in industrial application of food-related conversions and analyses[16]. For this reason, the ability to tolerate various chemicals is critical for a protease to expand its potential for use in the preparation of useful products. As shown in Table 2, reduced protease activity was observed in the presence of SDS (69.57%) and H_2O_2 (72.87%). Nevertheless, substantial activity still maintained (> 90%) in the presence of Tween 80 and Triton X-100. Different organic solvents exhibited different effects on protease activity (Table 2). Isoamylol and isopropanol can reduce the activity of the EH2. In addition, protease activity was increased obviously by methanol, ethanol and DMSO. This capability may be helping the EH2 for a promising candidate in water-organic solvent systems.

Table 2. Activity and Stability of EH2 in presence of surfactants, oxidizing agent and organic solvents

Detergents	Residual activity (%)
none	100
SDS (0.5%)	69.57 ± 1.55
Triton X-100 (1%)	91.86 ± 4.84
Tween 80 (1%)	104.94 ± 1.84
H2O2 (1%)	72.87 ± 1.55
Acetone (25% (v/v))	93.22 ± 5.72
dimethyl sulfoxide (DMSO) (25% (v/v))	128.08 ± 2.73
ethanol(25% (v/v))	110.21 ± 3.35

isopropanol(25% (v/v))	60.92 ± 2.46
methanol(25% (v/v))	115.49 ± 0.70
isoamyl alcohol(25% (v/v))	78.96 ± 2.73

2.3.4 *Hydrolysis of bovine serum albumin and insulin* B_{ox}

When the ratio of [E]/[S] was 1:163 in the enzymatic hydrolysis system for 0.5 h, 9 product peaks can be obtained. With the increase of enzymatic hydrolysis time, the peak of insulin Box decreased gradually (Figure 5A). And the number of product peaks did not increase substantially. It is possible that the abundance of some peaks was reduced due to the hydrolysis of larger peptides into smaller peptides. Proteases with more enzymatic cleavage sites would hydrolyze substrates more effectively, which contributed to expand its application fields.

After incubation for 20 min or 40 min, the protease could also hydrolyze BSA (containing 583 amino acids) into a plurality of peptides varying lengths (Figure 5B). In summary, EH2 with more enzymatic cleavage sites had a good hydrolysis effect on proteins with larger and smaller molecular weight and exhibited high-catalytic efficiency.

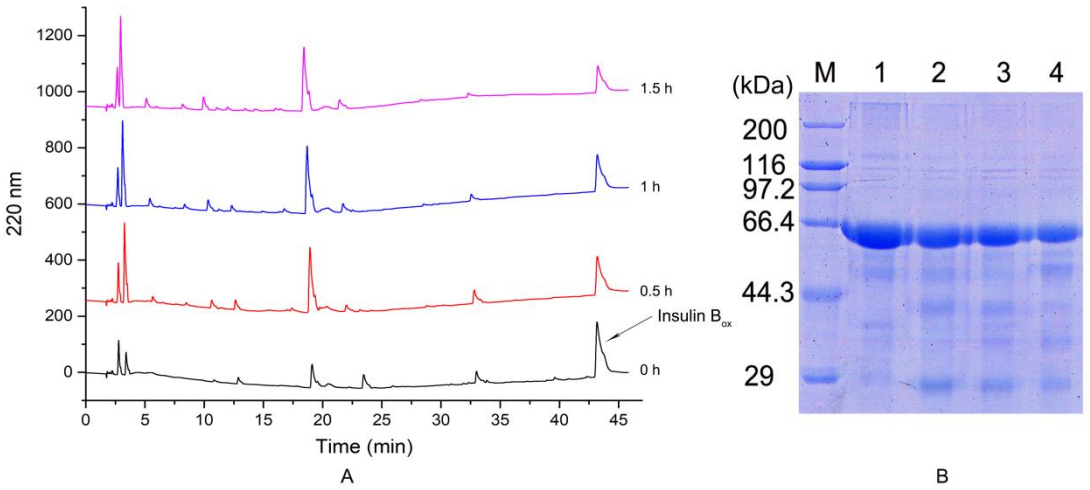

Figure 5. Hydrolysis of BSA and insulin B_{ox} by EH2. (A) Analysis of the hydrolysate of insulin Box by reversed-phase high performance liquid chromatography. (B) Analysis of the hydrolysate of BSA by SDS-PAEG. Line marked 1: 20 μL BSA with 1 μL EH2; line marked 2: 20 μL BSA was incubated with 1 μL EH2 for 40 min at 50℃; line marked 3: 20 μL BSA was incubated with 1 μL enzyme solution for 20 min at 50℃.

2.4 Mass Spectrum Identification of the protease

Mass spectrometry method was used for identification of purified EH2. The results were shown in Table 3. EH2 showed high sequence identity with metal protease from *Pseudoalteromonas* sp. A28 [17]. And the metal protease result was consistent with the conclusion obtained from the inhibitors experiments.

Table 3. Amino acid sequence of the protease by using NanoLC-ESI-MS/MS system

Peptide Mass	Peptide Sequence	Sequence Header	Similarity (%)	Mr (calc)
1491	^{386}GSNDWLVGQEIFK^{399}	metal protease [*Pseudoalteromonas* sp. A28]	100	35 kDa
1711	^{439}AFYNLATTPGWDTQK^{453}	metal protease [*Pseudoalteromonas* sp. A28]	100	
2093	^{386}GSNDWLVGQEIFKGNGALR^{405}	metal protease [*Pseudoalteromonas* sp. A28]	100	
2240	^{366}SGGLNEAFSDMAGEAAEFYMK^{386}	metal protease [*Pseudoalteromonas* sp. A28]	100	

2.5 Preparation of native collagen hydrolytic peptides

Collagen structure is defined by three almost identical polypeptide chains. Each polypeptide chain consists of repeating triplets $(Gly\text{-}X\text{-}Y)_n$ [18]. Animals' skins are a rich source of collagen with a variety of bioactivities. However, they are often discarded as by-products of the meat processing industries [10]. In this study, native collagens from porcine skins and salmon skins were both hydrolyzed with the EH2. The hydrolysis results of 10 min were analyzed by SDS-PAGE (Figure 6A) and the rate of hydrolysis was also measured (Figure 6B, 6C). Compared with porcine skins collagen, salmon skins collagen could be hydrolyzed to smaller pieces more effectively by EH2 with low enzyme concentrations. This result showed that the protease could be more suitable for the digestion of marine collagen. The higher the content of Hyp is, the stronger the ability of collagen to maintain the three-strand helical structure. And the content of Hyp in porcine collagen is significantly higher than that of fish. Thus, the porcine skins collagen is harder to hydrolyze. Of course, a large abundance of enzyme active sites may also one of the reasons for leaving to the

salmon skins collagen being more adequately hydrolyzed. After 4 h of treatment, the hydrolysis of native collagen degree did not increase significantly. This result indicated that the hydrolysis had reached maximum. It is essential for industrial enzyme to possess high enzymatic activity and substrate binding capability, which could shorten the reaction time and release antioxidant peptides more easily. In this respect, EH2 meet to our expectations.

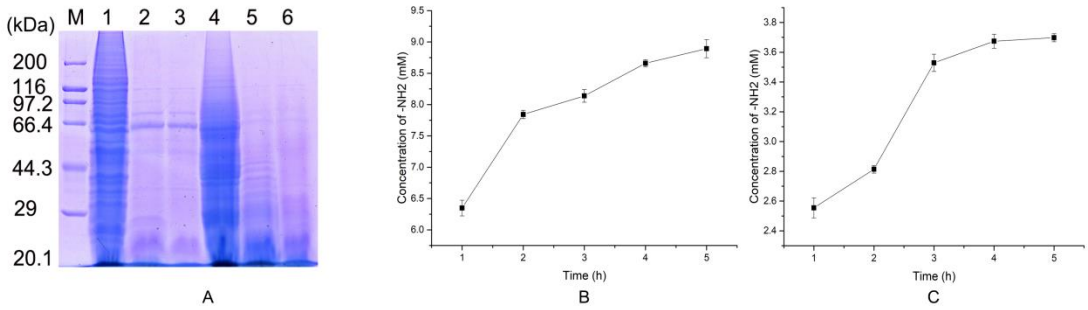

Figure 6. Hydrolysis of native collagen hydrolytic peptides. (A) The hydrolysis results of 20 min were analyzed by SDS-PAGE, Line marked 1: 20 μL porcine skins collagen (0.3 mg/mL) with 1μL EH2; Line marked 2: 20 μL porcine skins collagen (0.3 mg/mL) with 1μL EH2 for 10 min at 50℃; Line marked 3: 20 μL porcine skins collagen (0.3 mg/mL) with 1μL EH2 for 20 min at 50℃; Line marked 4: 20 μL salmon skins collagen (0.4 mg/mL) with 1μL EH2; Line marked 5: 20 μL salmon skins collagen (0.4 mg/mL) with 1μL EH2 for 10 min at 50℃; Line marked 6: 20 μL salmon skins collagen (0.4 mg/mL) with 1μL EH2 for 20 min at 50℃. (B) Hydrolysis degree of salmon skins collagen and (C) porcine skins collagen treated for 1, 2, 3, 4, and 5 hours.

2.6 Antioxidant Activity of the hydrolytic peptides

The performance of antioxidants often varies against different free radicals. DPPH radical scavenging, hydroxyl radical scavenging, and ORAC assay were used as measure for assessing antioxidant activities of each hydrolysate fractions of every

hour. As shown in the Figure 7, the DPPH scavenging activity of two hydrolytic products increased gradually with the time of hydrolysis until the 3 h time point, after which it began to/ decline. For salmon skins collagen hydrolysates, the strongest DPPH scavenging activity was 42.88%±1.85 (Figure 7A). The porcine skins collagen, by contrast, displayed persistent low DPPH scavenging activity during hydrolysis (Figure 7B). DPPH radical scavenging activity is generally used to determine hydrogen-donating ability of protein hydrolysates [19]. When DPPH encountered a hydrogen-donating substance, the radical would be scavenged, and the absorbance at 517 nm was reduced. Ours results suggested that the salmon skins collagen hydrolysates contained more electron donors (include amino acids or peptides), which could react with free radicals to convert them to much more stable products and terminate the radical chain reaction, than porcine skins collagen hydrolysates.

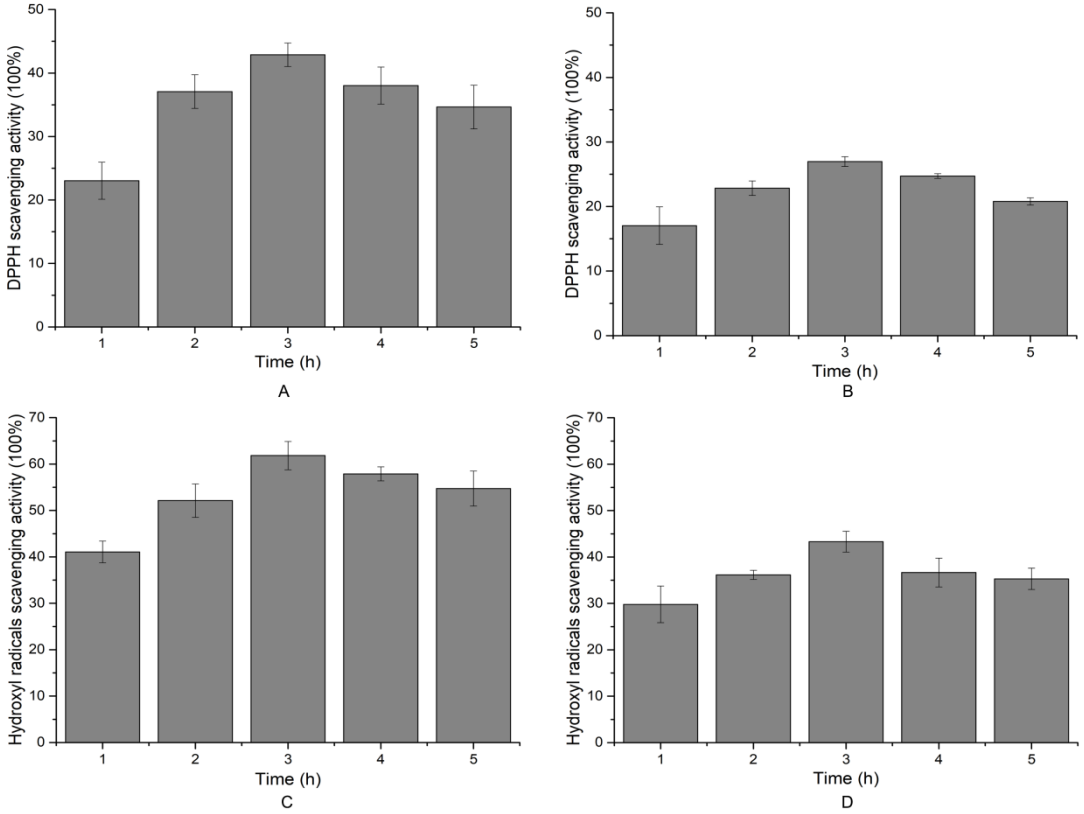

Figure 7. DPPH and hydroxyl radical scavenging activity of hydrolysates. (A) DPPH and (C) hydroxyl radical scavenging activity of salmon skins collagen hydrolysates. (B) DPPH and (D) hydroxyl radical scavenging activity of porcine skins collagen hydrolysates.

The highest hydroxyl radical scavenging ability of 61.83%±3.05 and 43.29%±2.25 were observed in the two hydrolysates collected at the 3 h time point, respectively (Figure 7C, 7D). The hydroxyl radical scavenging ability is a result from comprehensive action of the reducing power, donation of hydrogen and the scavenging of active oxygen [20]. The results suggest that two hydrolysates may provide good hydroxyl radical scavenging ability and help to offer a defensive shield against the hydroxyl radical [21].

In ORAC assay， the area under the fluorescence decay curve reflected the quantity of the peroxyl radical removed by peptides. As shown in Fig 8,

two hydrolytic products displayed its effect in decreasing the decay of fluorescence, and the porcine skins collagen hydrolysates antioxidant activity (7.72±0.13 μmol·TE/μmol) was much stronger than salmon skins collagen hydrolysates (4.15 ±0.05 μmol·TE/μmol). The result indicated that the porcine skins collagen hydrolysates might contain more active peptides working as hydrogen atom donors [22]. Meanwhile, hydrolysates showed a dose-dependent increase in the inhibition of fluorescence decay (Fig 8E, 8F).

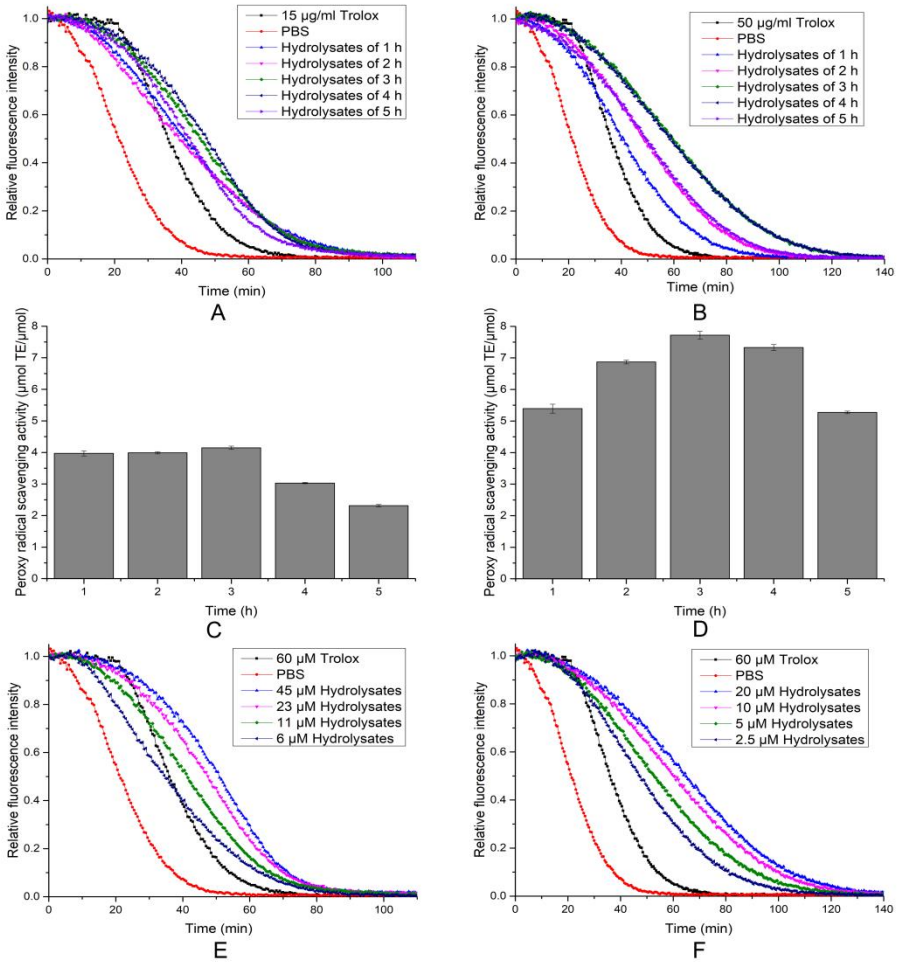

Figure 8. Peroxyl radical scavenging activity (ORAC assay) of hydrolysates. (A) (C) Peroxyl radical scavenging activity of salmon skins collagen hydrolysates with different time. (B) (D)Peroxyl radical scavenging activity of porcine skins collagen hydrolysates with different time. (E) Peroxyl radical scavenging activity of salmon skins collagen hydrolysates with different concentration. (F)Peroxyl radical scavenging activity of porcine skins collagen hydrolysates with different concentration.

The two hydrolysis products exerted the best effects on DPPH radicals, hydroxyl radicals and ORAC assay when hydrolyzed for 3 hours. After 3 hours the antioxidant activity began to decline. The reason may be that some of the antioxidant peptides were further hydrolyzed with increasing reaction time and low

molecular peptides contribute more to the inhibitory activity than polypeptides [12, 23]. In addition, DPPH and hydroxyl radical scavenging activity of salmon skins collagen hydrolysates were better than those of porcine skins collagen hydrolysates, but as for the absorption capability of against peroxyl radical, the latter had higher efficiency. The results indicated that hydrolysis time and type of substrate exerted important effects on the antioxidant property of hydrolysates. The ORAC of hydrolysates was not significantly correlated with the DPPH scavenging activity. Therefore, it is necessary to use more than one assay to measure the antioxidant capacity of protein hydrolysates. Antioxidant properties of peptides are greatly influenced by the amino acid composition [24], lengths[25] and hydrophobicity of the peptide [26]. Therefore, we conclude that the radical scavenging capacities of the hydrolysates might be due to the size and the hydrophobic amino acid content of the peptides and the concentration of electron-donating substances in the hydrolysates. Therefore, prepared hydrolysates with appropriate hydrolysis time and source could maximise their functions as radical scavengers.

2.7 Cytotoxicity and intracellular antioxidant activity of the hydrolytic peptidesof the hydrolytic peptides on HUVECs

Endothelial oxidative injury is a key event in the development of many diseases，such as diabetes, arteriosclerosis[27]. Resistance to oxidative damage of endothelial cells will be important in the prevention and treatment of various vascular dysfunction-related diseases. To evaluate the effects of the hydrolytic peptides of two collagens on HUVECs, MTT method was used. The results showed that the

hydrolytic peptides had no toxic to cells at 0.35-1.4 mM (Fig 9). In addition, it showed even higher cell viability in test groups. This confirmed that the hydrolytic peptides were safe and suitable for cell growth and can be used to ROS experiments.

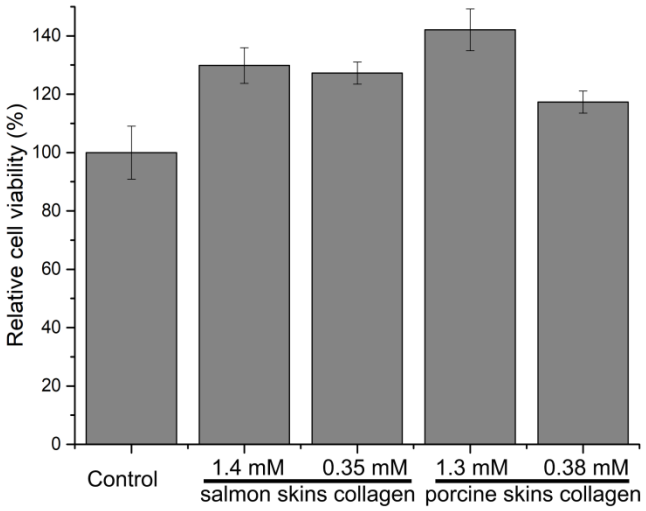

Figure 10. Cell viability determined by MTT assay. Values are expressed as the mean ± SD (n = 3).

High glucose could increase oxidative stress in peripheral tissues[28], and increased ROS generation was responsible for the stimulation by high glucose[29]. To investigate the intracellular ROS scavenging effects of the hydrolytic peptides, HUVECs were labelled with DCFH-DA. As shown in Figure 10, cells treated with 35 mM glucose displayed a stronger DCF-fluorescence intensity than cells in blank groups, which indicated that high glucose could increase oxidative stress in HUVECs. Cells treated with different concentrations of unpurified hydrolytic peptides also displayed low fluorescence intensity. Compared with the high-glucose group, even 1 nM mixed peptides can effectively reduce the levels of intracellular ROS. This results suggested that unpurified hydrolytic peptides attenuated the oxidative injury in

HUVECs. In addition, the intracellular radical-scavenging effects of 1 nM salmon skins collagen hydrolysates were better than those of porcine skins collagen hydrolysates. At present, research of antioxidant peptides mainly focuses on the purified peptides[30]. In fact, as a highly effective food additive, unpurified antioxidant peptides may be preferred because of the high cost and tedious operation of purification.

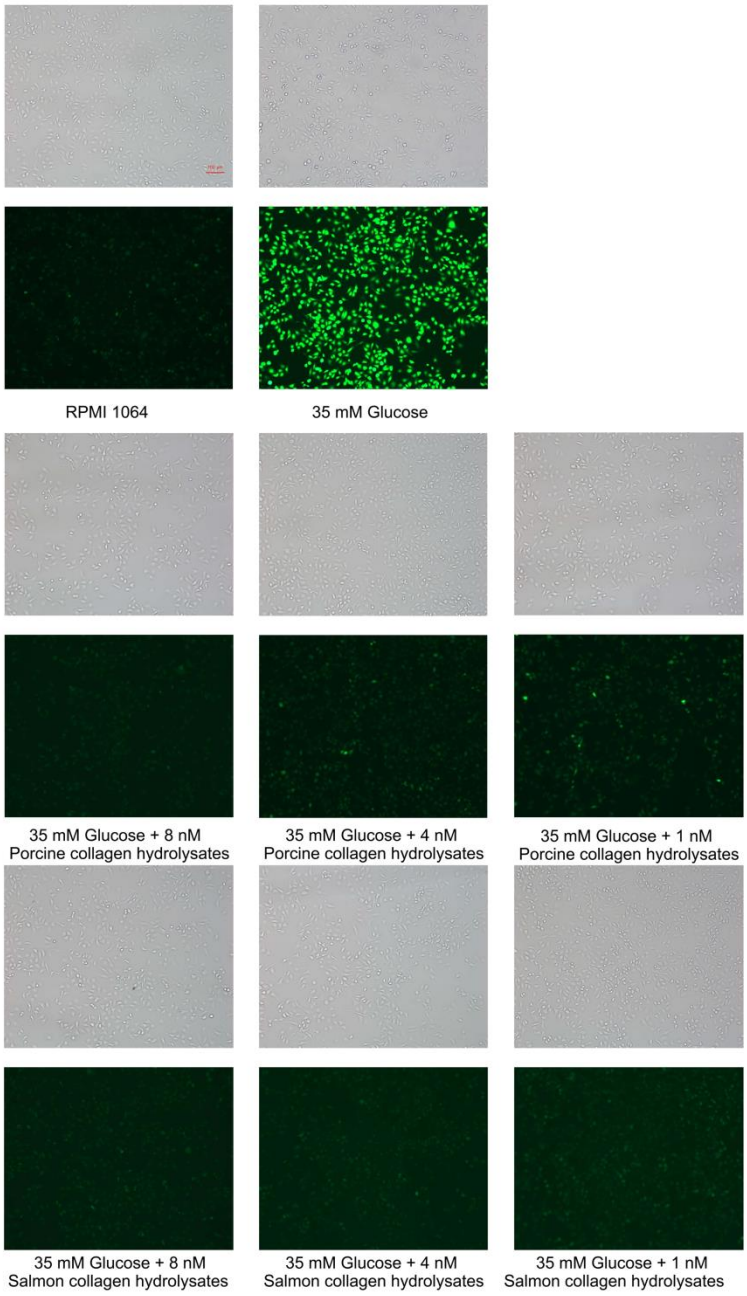

Figure 10. Intracellular ROS in HUVECs indicated as green fluorescence by DCFH-DA.

3. Materials and methods

3.1 Materials

Fresh salmon skins and porcine skins was purchased from seafood market, and was stored at -20℃ prior to use. The soybean meal, corn powder, and wheat bran were

purchased from supermarket. Tryptone and yeast extraction were purchased from OXOID (Basingstoke, UK). Sephadex 75 was purchased from GE Healthcare Life Sciences (Uppsala, Sweden). The other analytically pure regents used are commercially available.

3.2 Bacterial culture

The marine bacterial strains were isolated from the inshore environment of Bohai Sea and screened with a medium composed of 0.5% tryptone, 0.1% yeast extract, 0.5% casein, 1.5% agar powder, and artificial seawater (pH 7.8). The plates were incubated at 18℃ for 24 h. The formation of clear hydrolytic zone around the colonies was evaluated as proteolytic activity. All strains were maintained in 2216E agar medium and stored with 20% glycerol at -80℃.

3.3 Preparation of proteases

The strains were cultured at 18℃ with shaking at 200 rpm in 500 ml flasks with 50 ml fermentation broth[31]. The culture supernatant was collected by centrifugation (12,000 × g, 4℃, 30 min) after 84 h incubation and stored at -20℃ for the following experiment.

3.4 Protease assay

The protease activity of the culture supernatant to casein was detected by Folin phenol method. One unit of enzyme activity was determined as the amount of enzyme that

catalyzed the formation of 1 μg tyrosine per min. The experiment was repeated three times.

3.5 Protease purification

The crude enzyme of *Pseudoalteromonas* sp. H2 precipitated overnight with 40% $(NH_4)_2SO_4$ at 4℃. The precipitate was collected by centrifugation and dissolved in 20 mM Tris-HCl (pH 8), and dialyzed. The samples was loaded onto a 5 ml HiTrap™ DEAE column (GE Healthcare) previously equilibrated with 20 mM Tris-HCl (pH 8), then eluted with a NaCl gradient (0-1 M) in the same buffer at a flow rate of 1 ml /min. Active fractions were collected, concentrated by ultrafiltration (10 kDa MW cut-off membrane, Millipore), and subjected to gel filtration on Superdex 75 (10 × 300 Column 24 mL) previously equilibrated with 20 mM Tris-HCl (pH 8). The flow rate was 1 ml /min, and the active fractions were pooled for further analysis. Protein concentration was determined by BCA kit.

3.6 Zymography and SDS-PAGE analysis

The substrate immersing zymography method and SDS-PAGE was described previously [1]. The electrophoresis voltage of 5% stacking gel was 100 V, and 12% running gel was 150 V.

3.7 Characterization of the protease

3.7.1 *Effect of temperature and pH on protease activity and stability*

The optimal pH of the protease was determined by different buffers which pH ranging from 4 to 11 at 50℃ for 20 min. The buffers system were: citrate buffer (pH 4.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and Gly-NaOH buffer (pH 9.0 to 11.0). The effect of pH on protease stability was determined by measuring the residual activity after the enzyme was incubated with different pH buffers at 4℃ for 24 h. The optimal temperature was determined by performing the assay procedure at 40, 50, 60, 70 and 80℃. The thermal stability was determined by measuring the residual activity after protease was treated at 50, 60, 70℃ for 10, 20, 30, 40, 50, 60 min.

3.7.2 *Effect of inhibitors and metal ions on the protease activity*

The effect of metal ions was investigated by measuring the residual activity of the protease after pre-incubated with 2.5 and 10 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ag^{+}, Al^{3+}, Ba^{2+}, $Fe^{3+}$and Fe^{2+} for 30 min. 5 and 10 mM Phenyl Methane Sulphonyl Fluoride (PMSF), Ethylene Diamine Tetra Acetic acid (EDTA), and 1, 10-Phenanthroline (OP) were used for assessing the effect of protease inhibitors. The activity of samples without metal ions and inhibitors was set 100% activity as control.

3.7.3 *Effect of surfactants, oxidizing agents, and organic solvents on protease activity*

The effect of surfactants (SDS, Tween 80, and Triton X-100) and oxidizing agent H_2O_2 was also analyzed. The protease was pre-incubated with different agents for 1 h at room temperature, respectively. The compatibility of protease with organic solvents

was evaluated with acetone, dimethyl sulfoxide (DMSO), ethanol, isopropanol, methanol, and isoamyl alcohol (25% (v/v)). Protease was pre-incubated with organic solvents for 5 h at room temperature, and measured the protease residual activity.

3.8 Hydrolysis of bovine serum albumin and insulin chain B by the protease

The stock solution of insulin B_{ox}(10 mg/ml) was prepared by dissolving 100 μg insulin B_{ox}(Sigma-Aldrich) in 10 μl 0.01 M HCl. Insulin B_{ox} substrate(2 mg/ml) and enzyme solution (0.1 mg/ml) was mixed and incubated for a certain time at 45℃, and then 1 μL 10% Trifluoroacetic Acid (TFA) was added to terminate the reaction. The samples was loaded onto a C18 reversed-phase column previously equilibrated with 2% Methanol (contain 0.1% TFA), then eluted with a Methanol gradient (2%-100%) at a flow rate of 0.5 ml/ min, detection wavelength was 220 nm.

20 μL bovine serum albumin solutions (BSA, 1 mg/ml) with 1 μL enzyme solution (0.2 mg/ml) was mixed and incubated for 20 min and 40 min at 50℃, and electrophoresis after thermal denaturation.

3.9 Mass Spectrum Identification of the protease

The protease was identified by mass spectrometry. These proteins were excised from the polyacrylamide gel and digested in situ with trypsin, and the resulting peptide mixture was eluted from the gel. An aliquot of this tryptic peptide mixture was analyzed by Sangon Biotech Co.,Ltd.

3.10 Preparation of collagen hydrolytic peptides

Native collagen was extracted from porcine skins and salmon skins according to the method of Wu [32]. The protease (100 μL, 0.2 mg/ml) was mixed and incubated with native collagen (2 mL, 5 mg/ml) at 50℃ for 1 h. And Collagen hydrolytic peptides were collected. Free amino acid content was estimated by indene tri-ketone colorimetric method.

3.11 DPPH radical scavenging, Hydroxyl radicals scavenging and ORAC activity assay

The DPPH radical scavenging, hydroxyl radicals scavenging and ORAC activity assay was measured according to the method of Wu [30]. Mixed solution of hydrolytic peptides and DPPH (the ratio of hydrolysates to DPPH were 1:5) were sealed and incubated for 60 min at 37℃ in the dark, and the decrease was measured against ethanol in absorbance at 517 nm by Enspire spectrophotometer (Perkin Elmer, Waltham, MA, USA).

In hydroxyl radicals scavenging activity assay, FeSO4 solution (40 μl, 2 mM), 1, 10-phenanthroline (40 μl, 2 mM) and sample (80 μl) were mixed. Adding H_2O_2 (40 μl, 0.03% v/v) to initiate the reaction. The absorbance of the mix solution was measured at 536 nm after incubation at 37℃ for 60 min. The group without any antioxidant was used as the negative control, and the mixture without H_2O_2 was used as the blank.

In ORAC activity assay, sample solution (20 μL) and fluorescein (150 μL, 96 nM)

were added to a 96-well plate and pre-incubated at 37 ℃ in the Enspire spectrophotometer, and then AAPH (30 μL, 320 mM) was added to initiate the reaction. The reaction was performed at 37℃. The fluorescence intensity was measured every 60 s for 150 cycles with excitation and emission wavelengths of 485 nm and 538 nm, respectively. Vitamin E was used as a positive control.

3.12 The cytotoxicity of collagen hydrolytic peptides by MTT methods

Human umbilical vein endothelial cells (HUVECs) (1×10^5 cells/ml) were plated in 96-well plate and cultured in RPMI-1640 medium with 10% (v/v) foetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 for 12 h, then treated with different concentrations of hydrolytic peptides (0.35-1.4 mM) for 12 h. The effects of collagen hydrolytic peptides on the growth of cells were evaluated by a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay[30].

3.13 Determination of ROS level

The level of intracellular ROS (Reactive Oxygen Species) was determined by DCFH-DA(2，7-dichlorofuorescin diacetate). HUVECs (1×10^5 cells/ml) were cultured in 24-well plate for 12 h. Then, the medium was replaced by RPMI 1640 medium (without FBS, but with 35 mM glucose and different concentrations of peptides), and incubated for 12 h. Subsequently, RPMI 1640 medium with $1/10^3$ DCFH-DA (v/v) was added to each well and incubated for 1 h in cell incubator. Wash away excess DCFH-DA with 10 mM PBS, and images of stimulated HUVECs were

collected using a Nikon ECLIPSE TE2000-U with a digital CCD camera (DS-U2, Nikon, Japan) under fluorescence.

4. Conclusion

The study presented a report on the isolation of a metalloprotease protease from *Pseudoalteromonas* sp. H2. The protease with the abilities of high tolerance to pH, temperature and organic solvents was proved effective in preparation of antioxidant peptides by hydrolysis porcine and salmon skins collagen. Moreover, our results shown that DPPH and hydroxyl radical scavenging activity of salmon skins collagen hydrolysates were better than those of porcine skins collagen hydrolysates. And the intracellular radical-scavenging effects of the low concentration of hydrolysis, the former is also superior to the latter. However, as for the absorption capability of against peroxyl radical, the latter had higher efficiency. Hydrolysis time and type of substrate exerted important effects on the antioxidant property of hydrolysates. The performance of antioxidants often varies against different free radicals. The ORAC of hydrolysates was not significantly correlated with the DPPH scavenging activity. In all, the protease EH2 with multiple tolerance and efficient hydrolysis capacity indicating it could be a potential application in biocatalysis industry. In addition, the hydrolyzed peptides from collagen prepared by H2 have good antioxidant activity indicating it could be a potential additive in the food processing industry and cosmetics industry. Of course, in order to provide indications of potential health benefits, the in vivo studies are on the way.

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Conflict of interest The authors declare that they have no conflict of interest.

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